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Journal of Immunological Methods, 119 (1989) 255–258
Elsevier

JIM 05158

A rapid and direct method for the detection and quantification of interleukin-1 receptors using 96 well filtration plates

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(Received 26 October 1988, revised received 8 December 1988, accepted 13 December 1988)

A rapid method for the detection and quantification of interleukin-1 receptors in cultured cells has been developed. The receptor binding assay is carried out in sealed 96 well filtration plates. At the end of the incubation period the seal is removed and the cells are filtered under vacuum to separate free ligand from bound. After washing several times, individual wells are removed using a well punch and counted in a gamma counter. The method is rapid, accurate and capable of high sample throughput and should find wide application as a screen to evaluate IL-1-like drugs.

Key words: IL-1 receptor; Receptor screening; Binding assay

Introduction

Interleukin-1 (IL-1) is a polypeptide hormone that plays a major role in the control of a variety of immunologic and inflammatory events in the body (Dinarello, 1984). Increasing evidence suggests that IL-1 is associated with disease states like rheumatoid arthritis (Dinarello, 1984), and may also be involved in some forms of diabetes and atherosclerosis (Marx, 1988). Although the nature of the biochemical events that accompany IL-1 action are still poorly understood the first step is known to involve IL-1 binding to specific cell surface receptors (Dower and Urdal, 1987). Thus, the ability to antagonize IL-1 receptor binding either with drugs or with receptor antibodies represents a mean of ameliorating IL-1-linked disease states.

An automated radioreceptor assay capable of detecting and quantitating IL-1 binding sites would thus be extremely advantageous to rapidly screen IL-1-like drugs and potential IL-1 receptor antibodies from hybridoma supernatants. Receptor binding assays for IL-1 in cultured cells have been well described and are based on an oil centrifugation method to separate free from bound counts (Robb et al., 1984). Unfortunately the method is time consuming because the operator has first to pipette oil into centrifuge tubes, layer samples onto the oil, centrifuge the oil tubes and finally cut through the oil layer to collect the cell pellet for gamma counting. In addition the method suffers from sampling error which arise from the need to sample aliquots from the incubation mixture and transfer them to the oil tubes. Finally, the method is limited in its ability to handle large numbers of samples efficiently.

Thus, it is clear that existing IL-1 radioreceptor assays do not lend themselves well for the rapid screening of hybridoma supernatants or potential IL-1 antagonists. For this reason we have developed an IL-1 receptor binding assay on standard 96 well filtration plates, a format which is identical with that employed to grow hybridomas for

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monoclonal antibody production. The method is free from extensive manipulation, rapid, accurate, and importantly allows high sample throughout.

Materials and methods

Materials

Human recombinant IL-1β expressed in *E. coli* was purified to homogeneity according to methods previously described (Huang et al., 1987). ^{125}I-IL-1 (specific activity 143–180 μCi/μg), labeled using the Bolton-Hunter reagent, was from New England Nuclear. Reagents for electrophoresis were from Bio-Rad. Hepes and all other reagent grade chemicals were from Sigma.

Methods

Cell culture

Raji cells were obtained from the American Type Culture Collection and were maintained in RPMI 1640 medium containing 10% fetal calf serum. The cells were passaged weekly and the medium was changed two additional times weekly. For binding assays the cells were collected, washed three times with RPMI 1640 and resuspended in binding buffer (RPMI 1640 containing 1% bovine serum albumin, 20 mM Hepes pH 7.2). Cell viability was assessed by trypan blue exclusion and cell number was determined by counting the cells in a hemacytometer.

Receptor binding assay

Cell binding assays were carried out in 96 well STSV filtration plates (hydrophilic Durapore membranes, 5 μm pore size, Millipore, Bedford, MA) sealed with a microtiter plate sealer (Dynatech Laboratories), prior to setting up the binding assay, to prevent leakage. Raji cells (1×10^6 cells/well) in binding buffer were incubated with ^{125}I-labeled IL-1 (0.25 nM) and varying concentrations of unlabeled IL-1 for 3 h at 37°C. The microtiter plates were agitated on a microtiter agitator (Sarstedt TPM2) and the final incubation volume was 170 μl/well. At the end of the incubation the plate sealer was removed and the binding reaction was terminated by vacuum filtration. The wells were washed three times with 200 μl of ice-cold binding buffer using a 12 well automatic pipette. Individual wells were then punched out into counting vials using a Millipore filter punch, and counted in a gamma counter. Non-specific binding was determined in the presence of 1 μM unlabeled IL-1. The biologic activity of the radiolabeled IL-1 was at least 90% that of unlabeled IL-1 measured by a lymphocyte proliferation assay.

Data analysis

Equilibrium binding data were analyzed by Ligand (Munson and Rodbard, 1980) and modified for the IBM PC (McPherson, 1983).

Results and discussion

In order to develop a sensitive radioreceptor assay it is important to achieve a high signal-to-noise ratio. In vacuum filtration binding assays, which depend on a filter to separate free from bound counts, the filter composition is chosen so that it retains all of the bound counts and binds very little, if any, free radioligand. Thus, the first step in setting up an IL-1 receptor binding assay was to ensure that the filter chosen did not appreciable adsorb radiolabeled IL-1 in order to yield low background counts. For polypeptide hormones such as insulin and glucagon, nitrocellulose or Whatman glass fiber filters have proven to be suitable (Kahn et al., 1974; Desbuquois, 1985). Unfortunately, as can be seen in Table I both nitrocellulose and glass fiber filters are unsuitable for an IL-1 binding assay since they both adsorb high levels of IL-1, yielding an extremely high background that masks any specific binding. In contrast, Durapore membranes, which have a low protein binding capacity, gave a low background binding with IL-1. After subtraction of background, non-specific binding of IL-1 to the cells was around 9%, which compares favorably with binding studies reported using oil centrifugation (Horuk et al., 1987).

In order to determine the precision of the filtration assay we compared it to the oil centrifugation assay which is the standard protocol in use in most laboratories. Binding assays were carried out over a wide ligand concentration range and the

TABLE I

COMPARISON OF THE SEPARATION OF RECEPTOR BOUND IL-1 FROM FREE COUNTS USING A VARIETY OF DIFFERENT FILTER

Cells were incubated with IL-1 as indicated above, and the incubation was terminated by vacuum filtration through the appropriate filter as described below. A 'blank' tube containing radiolabeled IL-1 but no cells was also run.

Filter composition	Total binding (cpm)	Non-specific binding (cpm)	Blank (cpm)
Nitrocellulose	25063	16739	29036
Whatman GF/B	26947	15968	26046
Durapore	6813	1161	617

data were transformed according to Scatchard (1949). Scatchard analysis of equilibrium binding data for ^{125}I-IL at 37°C to Raji cells is shown in Fig. 1. The linear plots are indicative of a single class of IL-1 receptor binding sites as previously determined (Horuk et al., 1987). The calculated apparent K_D was 2.3 ± 0.1 nM which compares favorably with that obtained by the oil centrifugation method (Horuk et al., 1987). The microtiter binding assay has also been successfully used to assay IL-1 receptors in EL4 and 70Z/3 cells (data not shown).

On a technical note, it is extremely important that the bottoms of the filtration plates are sealed with a microtiter plate sealer prior to setting up the assay. Failure to do this results in spontaneous

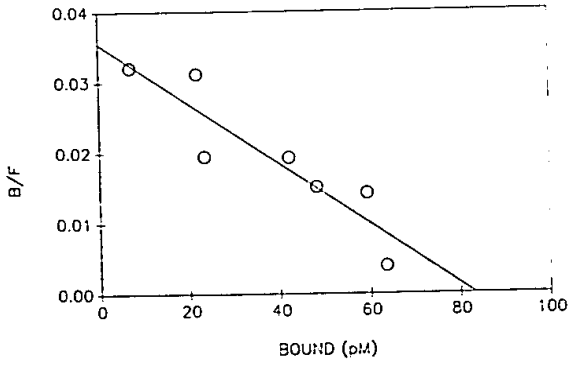

Fig. 1. Scatchard analysis of ^{125}I-IL-1 binding to Raji cells at 37°C. Cells (1×10^6) were incubated with varying concentrations of radiolabeled IL-1 for 3 h. Data were analyzed as indicated in the materials and methods section.

or 'gravity flow' of liquid during the course of the incubation. The variable well volumes resulting from this severely compromises the precision of the assay.

Since the affinity (K_D) of IL-1 binding to most cells is high, around 10 pM to 2 nM (Dower and Urdal, 1987; Horuk et al., 1987), washing of the bound cells to reduce background counts contributed by trapping should not pose a problem. In addition it has been demonstrated that the dissociation rate of IL-1 binding is fairly slow, in some cells greater than 7 h at 4°C, thus it is unlikely that many counts will be lost during the washing procedure. The filtration flow rate was extremely rapid, 5–30 s, and this ensures that little if any of the receptor bound counts will dissociate during either the initial filtration or during the subsequent washing.

The assay is reproducible at 4°C, however steady state binding is not achieved until after 6–7 h at this temperature. Binding experiments were carried out at 37°C because Raji cells internalize slowly at this temperature and steady state binding is achieved after only 3 h. Binding experiments that were carried out at 4°C in Raji cells incubated overnight for 20 h yielded kinetic constants that compared favorably to those obtained by conventional binding experiments using oil centrifugation.

The radioreceptor assay described here has several advantages over existing procedures. First, the 96 well plate format is identical to that employed for the growth of hybridoma supernatants. This makes it easy to transfer hybridoma supernatants efficiently and with a minimum of confusion. This should simplify procedures to obtain IL-1 receptor antibodies since a high sample throughput can be more readily achieved than with the oil centrifugation method. Second, the assay lends itself well for a drug screening program and should accelerate the progress made in obtaining potential IL-1 antagonists. Third, the assay would allow binding to IL-1 receptors in adherent cell lines to be analyzed in situ, without the need to detach cells by scraping followed by oil centrifugation, or by solubilizing in detergent followed by aspiration into counting vials. Finally, with the advent of multi-channel gamma counters capable of counting a 96 well format (Beta plate

counter, model 1205, LKB Instruments), together with attached automatic data handling programs, individual sample wells will not need to be processed and the filtration assay will be almost totally automated.

Acknowledgements

I would like to thank G. Sandlin and Sherrill Nurnberg for tissue culture, and Dianna Blessington for excellent technical assistance.

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